S100A8/A9 Activate Key Genes and Pathways in Colon Tumor Progression

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Abstract

The tumor microenvironment plays an important role in modulating tumor progression. Earlier, we showed that S100A8/A9 proteins secreted by myeloid-derived suppressor cells (MDSC) present within tumors and metastatic sites promote an autocrine pathway for accumulation of MDSC. In a mouse model of colitis-associated colon cancer, we also showed that S100A8/A9-positive cells accumulate in all regions of dysplasia and adenoma. Here we present evidence that S100A8/A9 interact with RAGE and carboxylated glycans on colon tumor cells and promote activation of MAPK and NF-κB signaling pathways. Comparison of gene expression profiles of S100A8/A9-activated colon tumor cells versus unactivated cells led us to identify a small cohort of genes upregulated in activated cells, including Cxcl1, Ccl5 and Ccl7, S10a9, Lcn2, Zc3h12a, Enpp2, and other genes, whose products promote leukocyte recruitment, angiogenesis, tumor migration, wound healing, and formation of premetastatic niches in distal metastatic organs. Consistent with this observation, in murine colon tumor models we found that chemokines were upregulated in tumors, and elevated in sera of tumor-bearing wild-type mice. Mice lacking S10A9 showed significantly reduced tumor incidence, growth and metastasis, reduced chemokine levels, and reduced infiltration of leukocytes and tumor cells in premetastatic organs. Studies using bone marrow chimeric mice revealed that S100A8/A9 expression on myeloid cells is essential for development of colon tumors. Our results thus reveal a novel role for myeloid-derived S100A8/A9 in activating specific downstream genes associated with tumorigenesis and in promoting tumor growth and metastasis.

Introduction

S100A8 and S100A9 belong to a family of more than 20 low-molecular-weight intracellular EF-hand motif calcium-binding proteins found exclusively in vertebrates (1–3). They are expressed predominantly by myeloid cells, including granulocytes, monocytes, myeloid-derived suppressor cells (MDSC), and other immature cells of myeloid lineage (4–7). Although the proteins are products of distinct genes, they are often coexpressed and function mainly as heterodimer of S100A8/A9 (calprotectin). Expression is downregulated during macrophage and dendritic cell differentiation (6, 8, 9), but can be induced in epithelial cells, osteoclasts, and keratinocytes (10). When these intracellular proteins are released into the extracellular medium in response to cell damage or activation they become danger signals (damage-associated molecular pattern molecules or DAMP), which alert the host of danger by triggering immune responses and activating repair mechanisms through interaction with pattern recognition receptors (11–15). Elevated S100A8/A9 is the hallmark of inflammatory conditions such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, cystic fibrosis and psoriasis (4, 11, 16). Critical roles for these proteins in endotoxin-induced lethality and systemic autoimmunity have recently been recognized (17, 18). In addition to expression within inflammatory milieu, strong upregulation of these proteins has also observed in many tumors, including gastric, colon, pancreatic, bladder, ovarian, thyroid, breast, and skin cancers (10, 19), and it is becoming increasingly clear that S100A8/A9 not only serve as markers of immune cells within the tumor microenvironment, but that they may also have independent pathogenic roles in cancer progression.

S100A8/A9 exhibit concentration-dependent dichotomy of function in tumors. At high concentrations (80–100 μg/mL), S100A8/A9 exert apoptotic effects on tumor cells (20), whereas at low concentrations (<25 μg/mL) promote tumor cell growth (21, 22). S100A8/A9 also stimulate tumor cell migration at low concentrations (23–27). More recently, other pathophysiological roles for these proteins in tumors have also been identified. Studies from our laboratories and others show that S100A8/A9 regulate the accumulation of MDSC (6, 7). MDSC are immature myeloid...
cells that expand during inflammation and in tumors and are potent suppressors of T-cell–mediated immune responses (28–30). Cheng and colleagues showed that tumor-derived factors promote sustained STAT3–dependent upregulation of S100A9 in myeloid precursors, which results in inhibition of differentiation to DC and accumulation of MDSC (6). We showed that S100A8/A9 are not only synthesized and secreted by MDSC, but they also have binding sites for S100A8/A9, and activate intracellular signaling that promote their migration (7). These findings strongly suggest that the S100A8/A9 proteins support an autocrine feedback loop that sustains accumulation of MDSC in tumors (31). S100A8/A9 are also involved in early metastatic processes. Expression of S100A8/A9 in myeloid and endothelial cells in premetastatic organs in response to soluble factors such as VEGF, TGFβ, and TNFα expressed by distal primary tumors promotes homing of tumor cells to premetastatic niches (24).

Recent studies argue for prominent roles for 2 pattern recognition receptors, TLR4 and RAGE, in S100A8/A9–mediated pathologic effects. The interaction with TLR4 promotes endotoxin-induced lethality and the development of systemic autoimmunity (17, 18). S100A9–mediated signaling through TLR4 also promotes premetastatic niches in lungs (32). Interaction of S100A8/A9 with RAGE has been shown to promote tumor growth, and MDSC migration (7, 21, 33). However, which receptor and signaling pathways are preferentially activated is not understood and may depend on the pathologic settings, cell types involved, ligand concentrations, and other factors. Distinct epitopes on RAGE and TLRs recognized by the ligands may also impart specificity. We found that S100A8/A9 bind to a subpopulation of RAGE expressing carboxylated glycans (22). These glycans show restricted expression on myeloid, endothelial, and tumor cells (34). Inhibiting carboxylated glycan–dependent interactions using mAbGB3.1, an anti-glycan antibody, blocked T-cell–mediated colitis (35), colitis–associated colon cancer (22), and accumulation of MDSC in a 4T1 model of metastatic mammary tumor (7). RAGE–deficient mice show reduced tumors in the colitis associated cancer (CAC) model (22) and inflammation–induced skin cancer model (33), suggesting that RAGE and carboxylated glycans form important components of tumor and stromal cells promoting molecular communications leading to myeloid accumulation and tumor growth.

Our previous observation of the presence of S100A8- and S100A9–positive myeloid cells in the microenvironment of colitis-induced colon tumors (22) prompted us to examine possible interactions of these proteins with tumor cells. We investigated S100A8/A9 binding to colon tumor cells and subsequent activation of signaling pathways and gene expression in vitro. The results led us to further investigate the contribution of these proteins in vivo to colon tumor growth, establishment of premetastatic niches in distal organs, and promotion of metastasis in tumor-bearing mice. Our findings uncovered several protumorigenic genes activated in tumor cells by S100A8/A9, strongly supporting a novel role of S100A8/A9 and myeloid cells in tumor progression.

Materials and Methods

Mouse and human S100A8 and S100A9 heterodimers and homodimers were purified as described (36) and rendered endotoxin-free. MC38 cells and MC38 cells stably expressing green fluorescent protein (GFP) were kind gifts from Drs. Ajit and Nissi Varki, University of California, San Diego. Caco-2 cells were obtained from American Type Culture Collection (ATCC). Cells were maintained in Dulbecco’s modified Eagle’s medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, glutamine, 10% FBS, 0.1 mmol/L nonessential amino acids, and 1 mmol/L sodium pyruvate (and 1 mg/mL G418 for MC38 cells expressing GFP).

Flow cytometric analysis

To detect surface expression of RAGE or carboxylated glycans, tumor cells were incubated with rabbit polyclonal anti-RAGE (raised against a peptide corresponding to amino acids 39–58 of human RAGE and which recognizes human, bovine, and mouse RAGE) or anti-carboxylated glycan antibody mAbGB3.1 (34) in HBSS containing 1% BSA, followed by Phycocerythrin-conjugated secondary antibodies and analyzed by flow cytometry with a FACScan (Becton Dickinson), equipped with CellQuest software, and gated by the side scatter and forward scatter filters.

Immunoprecipitation

MC38 or Caco-2 cells were isolated using PBS, containing 5 mmol/L EDTA, washed with HBSS, and incubated in HBSS medium containing purified mouse S100A8, S100A9, or S100A8/A9 for MC38 cells (at 1 μg/million cells in 100 μL final volume) or corresponding purified human proteins for Caco-2 cells for 1 hour at 4°C. Cells were washed with cold PBS twice, lysed in 20 mmol/L Tris-HCl, pH 7.4, with 150 mmol/L sodium chloride, 0.5% NP-40, and protease inhibitors, and centrifuged at 10,000 × g for 15 minutes to remove cell debris. Lysates were precleared with Protein G Sepharose beads for 1 hour at 4°C, and S100 proteins was immunoprecipitated by using rabbit polyclonal antibodies against the respective proteins or an irrelevant control antibody overnight at 4°C and Protein G Sepharose beads. The beads were washed of unbound proteins, and immunoprecipitated proteins were analyzed for RAGE or TLR4 by electrophoresis and Western blots as described in the following text.

siRNA treatment

A target-specific 20 to 25 nucleotides siRNA duplex designed to knock down expression of mouse S100A9 mRNA (calgranulin B siRNA; Santa Cruz Biotechnology Inc.) was used to transfect MC38 cells with transfection reagents and protocol provided by the manufacturer. Gene silencing was confirmed by Western blots of whole-cell
lysates with anti-S100A9. Cells were used for immunoprecipitation 48 hours after transfection.

**Signaling assays**

MC38 or Caco-2 cells were treated with mouse or human S100A8, S100A9, or S100A8/A9 (10 μg/mL) for 0, 15, 30, and 60 minutes, after overnight (16 hours) starvation in medium containing 0.1% serum. After indicated periods of incubation, cells were washed with cold PBS, harvested and lysed at 4°C, and lysates were analyzed by Western blot, using respective MAPK or IkB antibodies as described in the following text. For studies using mAbGB3.1 or anti-RAGE, following starvation, cells were preincubated for 2 hours with 20 μg/mL of mAbGB3.1 or rabbit polyclonal αRAGE prior to activation.

**Electrophoresis and Western blots**

Tumor cell lysates, immunoprecipitated proteins from tumor cells, or cell lysates from signaling assays were electrophoresed on denaturing and reducing 10% polyacrylamide gels and transferred to nitrocellulose membranes. The blots were blocked with 10% dry skimmed milk. To detect RAGE or TLR4 in lysates or immunoprecipitates, blots were incubated with goat polyclonal anti-mouse S100A9 or antimouse RAGE (R&D Systems), rat monoclonal anti-human RAGE (kind gift from Novartis foundation), or rabbit polyclonal anti-TLR4 antibody (Imgenex Corporation) followed by respective peroxidase-conjugated secondary antibody. Phosphorylation of ERK1/ERK2, p38, SAPK/JNK, and IkB in activated cell lysates was detected using respective rabbit polyclonal or mouse monoclonal phosphospecific antibodies (Cell Signaling Technology) followed by peroxidase-conjugated secondary antibodies. As loading controls, separate lanes with lysate proteins were incubated with rabbit polyclonal antibodies for total ERK1/ERK2, p38, SAPK/JNK, IkB, or β-actin (Cell Signaling Technology) followed by peroxidase-conjugated secondary antibody. Bands were visualized using ECL detection system (GE Healthcare).

**Measurement of NF-κB binding**

Nuclear extracts isolated from MC38 cells or Caco-2 cells treated with respective S100 proteins were assayed for NF-κB–p65 binding activity using TransAM NF-κB assay kit (ActiveMotif) according to the manufacturer’s instructions.

**Isolation of total RNA and gene expression profiling**

Subconfluent cultures of MC38 cells were serum-starved for 16 hours and activated with 10 μg/mL S100A8/A9 for 6 hours. Total RNA was extracted from unactivated or activated cells, using an RNeasy kit (Qiagen), and biotinylated cRNA was prepared using the Illumina RNA Amplification Kit (Applied Biosystems/Ambion). Hybridization to the Sentrix Mouse-6 Expression BeadChip containing more than 45,000 transcript-specific probe sequences/array (Illumina Incorporated), followed by washing and scanning, was performed according to manufacturer’s instructions. The resulting images were analyzed using GenomeStudio (Illumina Incorporated) and GeneSpringGX11 (Agilent Technologies) image processing software. Experiments were performed in duplicates.

**Real-time quantitative PCR analysis of chemokine genes**

SYBR Green oligonucleotide primers for the real-time quantitative PCR (qRT-PCR) analyses were designed using Primer 3 software. Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. Following RT using Roche Transcriptor First Strand cDNA synthesis kit (Roche Applied Science) PCR (45 cycles) was performed using Roche Lightcycler 480 as follows: preincubation at 95°C for 5 minutes, denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds, and elongation at 72°C for 10 seconds.

**Quantitation of CXCL1**

CXCL1 in culture supernatants and mouse sera were measured using a commercial ELISA kit (R&D Systems).

**Mouse tumor models**

S100A9 null mice were generated as described (37). They were backcrossed to C57BL/6 mice for more than 10 generations. Six to 8-week-old S100A9 null mice, their wild-type littermates, or age-matched wild-type mice were used for experiments. All animal protocols were approved by the Sanford-Burnham Medical Research Institute Animal Care and Use Committee and were in compliance with NIH policies.

**CAC model.** CAC was induced in separate groups of wild-type or S100A9 null mice, using AOM and DSS essentially as described (38) except that mice were subjected to 2 cycles of DSS 2 weeks apart. Animals were constantly monitored for clinical signs of illness and were sacrificed at the end of 2, 6, 12, or 20 weeks after DSS. Blood samples were collected by retro-orbital bleeding prior to induction of disease and at aforementioned time points. At each time point, colons were removed and fixed as “Swiss-rolls” in 4% buffered formalin. Stepwise sections were cut and stained with hematoxylin and eosin (H&E). Colonic inflammation, dysplasia, and neoplasms were graded on the basis of described criteria (38).

**CAC in bone marrow chimeric mice.** Bone marrow cells were aseptically isolated from the femur and tibia of wild-type or S100A9 null mice and each injected intravenously into either wild-type or S100A9 null mice at 6 million cells per mouse. Recipient mice were lethally irradiated using a Gammacell 40 Exactor (9 Gy from a 137Cs source) before injection. Reconstitution of leukocyte populations was comparable in these groups. We confirmed successful engraftment by measurement of S100A8/A9 in serum. Mice were subjected to the AOM/DSS protocol 4 weeks later, sacrificed 12 weeks after DSS, and blood and tissues were collected.

**MC38 ectopic tumor model.** To generate primary tumors, single-cell suspensions of 1 × 10⁶ MC38 cells in logarithmic phase of growth were injected subcutaneously.
into the flank of wild-type or S100A9 null mice and allowed to grow for 10 to 20 days. To evaluate the role of carboxylated glycans, separate groups of wild-type mice were treated with 10 μg/gm of mAbGB3.1 weekly starting from 2 days prior to injection of tumor cells. Tumor growth was measured using calipers over the experimental period, and tumor volume was estimated. Lungs, liver (primary metastatic organs), and tumors were frozen for further analysis. Bone marrow responses to tumor growth were evaluated as follows: Bone marrow cells were isolated from femur and tibia of mice and RBCs lysed according to standard protocols. Myeloid cells were stained with differentiation marker CD11b and costained with mAbGB3.1, anti-RAGE, or anti-Gr-1 (Ly6C and Ly6G; BD-Pharmin-gen). 7-Aminoactinomycin D (7-AAD; Invitrogen) or propidium iodide (BD-Pharmin-gen) was included to identify dead cells and analyzed by flow cytometry. Peripheral blood hematology profile was obtained on EDTA samples, using a VetScan HMII hematology system (Abaxis).

Liver metastasis model. S100A9 null mice and age-matched wild-type C57BL/6 mice were anesthetized using i.p. injection of avertin. Under aseptic conditions, a small incision was made in the left upper flank to visualize the spleen, and 1 × 10^6 MC38 cells in 50 μL of serum-free medium were injected under the spleen capsule with a 27-gauge needle. The spleen was then inserted back into the abdominal cavity and the peritoneum and abdominal walls were sutured with silk. Animals were sacrificed 2 weeks later, and livers were isolated, fixed in buffered formalin, and paraffin-embedded. Tumors were enumerated by visual inspection and by examining liver sections stained by H&E. Slides were scanned using an automatic high-throughput ScanScope, viewed, and tumor areas were measured using Aperio software (Aperio).

Immunosuppression assay

Spleen CD4^+ T cells isolated from OTII transgenic mice (kindly provided by the Rickert and Bradley labs, Sanford-Burnham Medical Research Institute) were cocultured in 48-well plates at 37°C in RPMI-1640 medium (containing 10% FBS, penicillin, streptomycin, and 2-ME) with 48-well plates at 37°C in RPMI-1640 medium (containing 10% FBS, penicillin, streptomycin, and 2-ME) with CD11b^+Gr1^+ cells isolated from the spleens of tumor-bearing mice by MACS, at increasing ratios of MDSC:T cells in the presence of 10 μg/mL OVA peptide (OVA323-339). Cells were pulsed with 1 μCi [3H]thymidine/well on day 3, and 18 hours later, the cells were harvested and counted. Proliferation in the absence of MDSC was considered 100%.

Immunohistochemical analysis

Swiss rolls of colons from the AOM/DSS model were deparafinized and endogenous peroxidase blocked by incubating with 0.36% beta-glucose, 0.01% glucose oxidase, and 0.013% sodium azide in PBS for 60 minutes at 37°C. The sections were stained with 1:50 dilution of anti-mouse CXCL1, or CCL7 (Santa Cruz Biotechnology), followed by goat-anti-peroxidase (1:100) and developed using DAB substrate. To characterize macrophage populations, frozen sections of tumors and premetastatic livers and lungs from the MC38 tumor model were stained with 1:50 dilution of anti-mouse CD11b and anti-mouse Gr-1 (BD-Pharmin-gen) followed by Alexa-488- and Alexa-594-conjugated secondary antibodies (Invitrogen), and cover-slipped with VectaShield 4’,6-diamidino-2-phenylindole (DAPI) mounting medium (Vector Laboratories). MC38 tumor sections were also separately blocked with glucose oxidase as before and stained with anti-mouse S100A9 (R&D Systems), anti-mouse CD31 (BD-Pharmin-gen), or F4/80 (Invitrogen), followed by 1:100 dilution of respective peroxidase-conjugated secondary antibodies and developed with DAB substrate. Slides were scanned using an automatic high-throughput ScanScope and viewed using Aperio software. They were also examined using an Inverted TE300 Nikon Wide Field and Fluorescence Microscope and images were acquired with a CCD SPOT RT Camera (Diagnostic Instruments Inc.), using SPOT advanced software.

Statistics

Statistical comparisons were performed using paired t test, and P values were calculated using GraphPad Prism. Differences were considered statistically significant when P < 0.05.

Results

RAGE is the receptor for S100A8/A9 on colon tumor cells

We earlier found S100A8/A9^+CD11b^+Gr1^+ myeloid progenitor cells in the tumor microenvironment of colitis-induced colon tumors, whereas they were absent from the normal adjacent colon tissue (22). Our subsequent study in a 4T1 model of mammary carcinoma showed that these were MDSC (7). Given that activated myeloid cells and MDSC can secrete S100A8/A9, we postulated that there might be cross-talk between myeloid cells and colon tumor cells and that S100A8/A9 might interact with the tumor epithelium. In support of this, we earlier found that CT-26 mouse colon tumor cells expressed binding sites for S100A8/A9 and that a subpopulation of RAGE expressed on these cells is modified by carboxylated glycans (22). The proteins have been earlier shown to bind to RAGE on human prostate and breast cancer cells (21, 23). To further study cell-signaling pathways mediated by S100A8/A9 interactions, in this study, we used MC38 colon tumor cells that are syngeneic to C57BL/6 strain, as S100A9 null mice and RAGE null mice have been backcrossed to this strain. We first confirmed cell surface expression of RAGE and carboxylated glycans on MC38 colon tumor cells by flow cytometry (Fig. 1A). Examination of whole-cell lysates also showed that MC38 cells constitutively express both RAGE and TLR4 (Fig. 1B and C). To investigate whether RAGE or TLR4 provided binding sites for S100A8/A9 on MC38 colon tumor cells, we performed coimmunoprecipitation assays. Lysates from MC38 cells incubated with S100A8 or S100A9 homodimers or S100A8/A9 heterodimers.
heterodimer were immunoprecipitated with anti-S100A8 or anti-S100A9 antibodies and the immunoprecipitated proteins were separated by electrophoresis and immunoblotted with anti-RAGE or anti-TLR4. We could only detect RAGE in lysates of cells incubated with S100A8 or S100A9 homodimers or the heterodimer but did not detect TLR4 (not shown). These results suggested that RAGE could be the predominant receptor for S100A8/A9 on colon tumor cells.

**RAGE and carboxylated glycan-dependent binding of S100A8/A9 promotes MAPK and NF-κB signaling**

As RAGE ligation activates all members of the MAPK cascades, including the p38, ERK, and the JNK families, and promotes NF-κB activation (13), we next examined S100A8/A9 activated signaling pathways in colon tumor cells. We analyzed lysates of MC38 cells stimulated with low concentrations (10 μg/mL) of S100A8/A9 for varying periods of time by immunoblotting, using specific phospho-MAPK antibodies. This concentration was chosen for stimulation, as our earlier studies and that of Ghavami and colleagues show that S100A8/A9 at 1 to 10 μg/mL induced tumor cell growth (21, 22). Ghavami and colleagues also showed that S100A8/A9 at 10 μg/mL stimulated intracellular signaling in human breast cancer cell lines. In tumor-bearing mice, we found serum levels of S100A8/A9 in the order of approximately 500 ng/mL (7). However, local concentrations in inflamed and tumor tissues could be several micrograms per milliliter, for example, as shown in exudates of carrageenan-induced inflammation, where S100A9 levels are reported to be approximately 1 to 4 mg/mL (39).

S100A8/A9 stimulated rapid phosphorylation of ERK1/ERK2 and SAPK/JNK in MC38 cells within 15 minutes, with reduced but sustained phosphorylation up to 60 minutes, whereas there was no detectable phosphorylation of p38 (Fig. 2A). This effect was different from RAGE-dependent, S100A8/A9-induced activation triggered in human prostate and breast cancer cells (21, 23), where ERK1/ERK2 and p38 are activated but not SAPK/JNK, suggesting that S100A8/A9 preferentially activate different MAPK pathways depending upon tumor cell type. Phosphorylation of ERK1/ERK2 was also seen in Caco-2 human colon tumor cells (Fig. 2B). ERK1/ERK2 phosphorylation was inhibited in both MC38 and Caco-2 cells when they were preincubated with mAbGB3.1 or anti-RAGE, suggesting that the effects were mediated through RAGE and carboxylated glycans (Fig. 2B). Because S100A8 and S100A9 homodimers also bind to RAGE, we investigated whether homodimers would individually stimulate activation. We found that S100A8 and S100A9 homodimers also bind to RAGE, and S100A8/A9 stimulated a more delayed activation of ERK1/ERK2 in MC38 cells when compared with the heterodimers (Fig. 2C).

S100A8/A9 also induced phosphorylation of IκBα in MC38 and Caco-2 cells within 30 minutes (Fig. 3A),...
These results indicated that RAGE and carboxy-}

...pretreated with mAbGB3.1 or anti-RAGE IgG... This effect was significantly inhibited when

...stimulated cells compared with unstimulated... found considerable NF-

...strength of the inducer (41).... This has been attributed to impaired stimula-

...incomplete IκBα degradation following stimulation, despite evidence for concomitant IκBβ phosphorylation and NF-κB activation (40). This has been attributed to impaired stimulation of an upstream IKK activator, and an altered steady-state level of IκBβ, which is dependent on rate of resynthesis and strength of the inducer (41).

...further confirm S100A8/A9-induced activation of NF-κB pathway, we examined nuclear extracts isolated from MC38 and Caco-2 colon tumor cells and found considerable NF-κB–p65 in the extracts from S100A8/A9 stimulated cells compared with unstimulated cells (Fig. 3B). This effect was significantly inhibited when cells were pretreated with mAbGB3.1 or anti-RAGE IgG (Fig. 3B). These results indicated that RAGE and carboxy-

**Stimulation of colon tumor cells by S100A8/A9 promotes protumorigenic gene expression**

MAPK pathways link extracellular signals with intracellular responses promoting cell growth, proliferation, differentiation, and migration (42). Activation of NF-κB–dependent genetic programs in tumor cells and macrophages is critical for development of inflammation-based tumors (43–45). We therefore reasoned that gene expression studies of activated colon tumor cells might provide valuable insight into the consequences of S100A8/A9 activation. To identify whether S100A8/A9-activated signaling pathways promoted gene transcription, we isolated total RNA from S100A8/A9 stimulated and unstimulated MC38 colon tumor cells and performed global gene expression analysis. Surprisingly, we found only a small cohort of 28 differentially expressed genes (P < 0.01), of which, 24 were upregulated and 4 were downregulated in stimulated cells compared with unstimulated cells (Fig. 4A). Of these, the expression of 14 genes was 2-fold or more when compared with nonstimulated cells. Most of the upregulated genes encoded proteins with either well-known or more recently recognized functions in leukocyte migration and recruitment, inflammation, proliferation of tumor cells, tumor invasion, angiogenesis, and wound healing (Table 1). These genes included 4 chemokines (Cxcl1, Ccl5, Ccl2, Ccl7), NF-κB family member (Nfkbia), zinc transporter (Slc39a10 or Zip10), lipocalin-2 (Lcn2), Ectonucleotide pyrophosphatase/phosphodiesterase family member 2 (Enpp2), zinc finger protein (Znfh12a), guanylate binding protein 4 (Gbp4), anti-leukoproteinase (Slpi), proliferin-2 (plf2), and apoptotic gene Fas. Many are upregulated in human tumors, their expression correlating with poor prognosis (46–51). qRT-PCR analysis of chemokine genes Cxcl1, Ccl5, and Ccl7 further confirmed elevated transcript levels in S100A8/A9-stimulated cells (~6-fold increase in Cxcl1, 2-fold increase in Ccl5, and 2.5-fold increase in Ccl7) compared with unstimulated cells (Fig. 4B). We next examined whether upregulation of the Cxcl1 gene in cell cultures stimulated with S100A8/A9 led to corresponding secretion of CXCL1 (GROα or KC) protein. We analyzed culture supernatants of MC38 cells stimulated with S100A8/A9 for varying periods of time for CXCL1 by ELISA. We found that unstimulated MC38 cells constitutively secreted CXCL1 (Fig. 4C). In addition, in line with the gene expression data, S100A8/A9 activation led to a 3-fold increase in secretion of CXCL1 compared with unstimulated cells within 6 hours of stimulation (Fig. 4C). This S100A8/A9-induced secretion was significantly diminished when cells were pretreated with anti-RAGE or mAbGB3.1.

**Mouse models of colon tumors**

Our earlier studies and those of Cheng and colleagues have shown that S100A8 and S100A9 play a critical role in...
tumor growth and metastasis through increased accumulation MDSC (6, 7). A role in growth and migration of tumor cells has also been described by many studies (21–27).

However, molecular signature of S100A8/A9-activated cells revealed by gene expression analysis as shown previously strongly implied that RAGE and carboxylated glycan–dependent activation of tumor cells by S100A8/A9 differentially altered expression of genes whose products could mediate many protumorigenic effects, predicting that S100A8/A9 could have other novel roles in tumor progression. To further elucidate protumorigenic and pro-metastatic roles of S100A8/A9 in vivo, we subjected S100A9 null mice to different colon tumor models and compared responses with those observed in wild-type mice. Deletion of S100A9 in mice leads to a complete lack of S100A8 and a functional S100A8/A9 complex in cells of peripheral blood and bone marrow, despite normal mRNA levels of S100A8, suggesting that S100A9 expression is important for the stability of S100A8 protein (37, 52). Induction of tumors in S100A9 null mice thus provided us an excellent opportunity to test the importance of both proteins in tumorigenesis and malignancy.

Reduced tumor incidence and chemokine expression in S100A9 null mice in the CAC model

In the CAC model, we induced chemokine expression in S100A9 null mice and wild-type mice by azoxymethane (AOM) injection followed by 2 cycles of dextran sodium sulfate (DSS) treatment as described earlier (22). DSS causes epithelial damage and triggers an innate immune response that recruits activated macrophages and induces an acute colitis evident within 2 weeks after DSS. This initial response progresses to chronic inflammation by about 6 weeks by activation of adaptive immune responses (53). Wild-type mice develop dysplasia, adenoma, and adenocarcinoma within 12 to 20 weeks of combined administration of AOM and DSS (43, 54–56) with 100% penetrance. Both S100A9 null mice and age-matched C57BL/6 wild-type mice lost up to 10% of body weight after DSS treatment before recovery, and colons showed inflammation in both S100A9 null mice and wild-type mice (not shown), suggesting that S100A8/A9 do not contribute to DSS-induced colon inflammation. However, there was a significant reduction in tumor incidence in S100A9 null mice at 12 and 20 weeks after AOM/DSS (Fig. 5A and B). In contrast, all the wild-type mice developed adenomas (5–8 tumors per mouse) by 12 to 20 weeks, with a few adenocarcinomas by 20 weeks, suggesting that S100A8/A9 could exert independent roles in the tumorigenic phase of CAC.

We had earlier shown that S100A8/A9þ and CD11bþ/Gr1þ myeloid cells infiltrate all regions of dysplasia and tumors in this model (22). Because chemokines are upregulated in colon tumor cells in vitro in response to S100A8/A9, we examined whether chemokines CXCL1 and CCL7 were also induced in the colon tumors in this model. We found moderate to intense staining for CXCL1 and CCL7 in most epithelial and some stromal cells in tumor regions but not in adjacent normal tissues. Staining was reduced in tumor regions from S100A9 null mice (Fig. 5C). We also measured serum CXCL1 as a marker of S100A8/A9–induced activation of tumor cells. Serum CXCL1 was elevated 2- to 3-fold compared with pretumor levels in all wild-type mice, at 12 weeks of disease initiation, when the tumors are not invasive, whereas CXCL1 levels were minimally altered in tumor-bearing S100A9 null mice (Fig. 5D). This further substantiated our in vitro findings that S100A9/A9 promoted expression of protumorigenic downstream effectors in early tumors.

Bone marrow–derived cells in the tumor microenvironment contribute S100A8/A9

Epithelial cells can express S100A8/A9. To investigate whether S100A8/A9 expressed by tumor cells or infiltrating bone marrow–derived myeloid cells within the tumor...
microenvironment is required for disease progression, we evaluated tumorigenesis in chimeric mice after bone marrow transplantation. Bone marrow cells from wild-type or S100A9 null mice were injected into lethally irradiated groups of recipient wild-type or S100A9 null mice. Chimerism was confirmed by measurement of S100A8/A9 in serum (not shown). Mice were subjected to the AOM/DSS protocol 4 weeks later and sacrificed 12 weeks after initiation of disease. Tumor incidence in wild-type mice reconstituted with wild-type bone marrow cells (WT → WT) and S100A9 null mice reconstituted with S100A9 null bone marrow cells (S100A9 null →
S100A9 null) was similar to responses seen earlier in wild-type mice and S100A9 null mice (Fig. 5E). However, S100A9 null mice reconstituted with bone marrow cells from wild-type mice (WT → S100A9 null) showed higher incidence of tumors than wild-type mice reconstituted with bone marrow cells from S100A9 null (S100A9 → WT) mice. This strongly indicated that S100A8/A9 expressed by bone marrow–derived cells in the tumor microenvironment is essential for the promotion of tumorigenesis.

Reduced MC38 colon tumor growth and metastasis in S100A9 null mice

The CAC model described previously allows us to understand the role of S100A8/A9 in early events in colon carcinogenesis under the setting of inflammation. However, the tumors rarely became invasive and malignant within the experimental period of 20 weeks. Therefore, to define the role of S100A8/A9 and its downstream effectors in tumor invasion, myeloid cell migration, and formation of premetastatic niches in distal organs, we used a primary ectopic tumor model using MC38 colon tumor cells. We followed tumor growth in wild-type and S100A9 null mice injected s.c. with 1 × 10⁶ MC38 cells. Tumors were evident in all wild-type mice (n = 10) by 7 to 10 days after injection and continued to grow until 21 days when the mice were sacrificed. When S100A9 null mice were challenged with MC38 cells, tumors were significantly smaller in 6 of 12 S100A9 null mice at 21 days after injection (Fig. 6A). In addition, tumors were completely rejected in 2 of the remaining 6 S100A9 null mice. Collectively, 8 of 12 S100A9 null mice (67%) examined showed minimal tumor growth or tumor rejection. Tumor growth in wild-type mice was accompanied by elevated serum CXCL1 levels but not in S100A9 null mice (Fig. 6B).

Because CXCL1 promotes MDSC and other myeloid cell recruitment within tumors and premetastatic organs, we measured bone marrow responses to the ectopic MC38 tumors and found significantly increased CD11b⁺ populations coexpressing Gr1, carboxylated glycans (as stained by mAbGB3.1), or RAGE in all of the tumor-bearing wild-type mice at 18 to 21 days after transplantation compared with tumor-free control mice (Fig. 6C). CD11b⁺ Gr1⁺ cells were also found within the tumors and substantially reduced in tumors from S100A9 null mice, whereas the levels of F4/80⁺ macrophages and CD31⁺ endothelial cells were unchanged, suggesting that S100A8/A9 do not alter intra-tumoral infiltration of other tumor-associated, angiogenic macrophages, while affecting infiltration of CD11b⁺ Gr1⁺ cells (Fig. 6D). To confirm that these were in fact MDSC, we isolated CD11b⁺ Gr1⁺ cells from the spleens of MC38 tumor-bearing mice and cocultured them at varying ratios

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### Table 1. Protumorigenic genes activated in colon tumor cells by S100A8/A9

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Known functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cxcl1</td>
<td>NM-008176.1</td>
<td>Chemokine, promote angiogenesis, mobilization of leukocytes including MDSC</td>
</tr>
<tr>
<td>C3</td>
<td>NM-009778.1</td>
<td>Complement component 3, mobilization of HSC into tumor stroma</td>
</tr>
<tr>
<td>Slc39a10</td>
<td>NM_172653.2</td>
<td>Zinc transporter, belongs to Zip family (Zip10), upregulated in endometrial carcinoma, promote migration of breast tumor cells IlkB family, IL-6 activator, modulates NF-κB transcription</td>
</tr>
<tr>
<td>Nfkbiz</td>
<td>NM_030612</td>
<td></td>
</tr>
<tr>
<td>Lcn2</td>
<td>NM-008491.1</td>
<td>Lipocalin 2, upregulated in inflammation, has pro- and antitumor effects</td>
</tr>
<tr>
<td>Fas</td>
<td>NM_007897.1</td>
<td>Apoptosis, TNF family member with the death domain</td>
</tr>
<tr>
<td>Zc3hi2a</td>
<td>NM_153159.1</td>
<td>Zinc finger family, RNase, controls stability of inflammatory genes, mediates CCL2 induced angiogenesis, macrophage activation</td>
</tr>
<tr>
<td>Enpp2</td>
<td>NM_015744.1</td>
<td>Ectonucleotide pyrophosphatase/phosphodiesterase family member 2, autotaxin, promotes tumor cell migration (invasion), angiogenesis, and its expression is upregulated in several kinds of carcinomas</td>
</tr>
<tr>
<td>Ccl5</td>
<td>NM_013653.1</td>
<td>Chemokine, stimulate angiogenesis</td>
</tr>
<tr>
<td>Ccl2</td>
<td>NM_013653.2</td>
<td>Chemokine, potent stimulator of angiogenesis</td>
</tr>
<tr>
<td>Ccl7</td>
<td>NM_013653.3</td>
<td>Chemokine, promote macrophage infiltration into tumors</td>
</tr>
<tr>
<td>Gbp4</td>
<td>NM_018734.2</td>
<td>Guanylate binding protein 4; gene family induced during macrophage activation, IFN gamma inducible, GTPase</td>
</tr>
<tr>
<td>Slpi</td>
<td>NM.011414.1</td>
<td>Secretory leucocyte peptidase inhibitor, upregulated in tumors, secreted inhibitor, which protects epithelial tissues from serine proteases, implicated in wound healing</td>
</tr>
<tr>
<td>Pif2</td>
<td>NM.011118.1</td>
<td>Proliferin-2, HSC growth factor, associated with angiogenesis and wound healing</td>
</tr>
</tbody>
</table>

NOTE: From refs. 47–51, 66–78.
with CD4+ T cells from OTII transgenic mice and OVA peptide (OVA323-339) and measured T-cell proliferation by uptake of [3H]thymidine. With increasing ratios of MDSC: T cells, splenic CD11b+ Gr1+ cells from tumor-bearing mice progressively reduced T-cell proliferation (Supplementary Fig. S2).

A more recent study by Connolly and colleagues shows that the expansion of CD11b+ Gr1+ MDSC in premeta-
static sites in liver in response to intraabdominal tumors is contingent upon the expression of CXCL1 (57). In keeping with observation, and with elevated serum CXCL1 levels, we found markedly increased accumulation of CD11b+ /Gr1+ cells in the premetastatic lungs and liver of tumor-bearing mice 21 days after tumor initiation, compared with tumor-free mice (Fig. 6E, shown for liver). To exclude the possibility that any micrometastasis of MC38 cells in liver

**Figure 6.** A, tumor volumes of ectopic MC38 tumors in wild-type (n = 10) and S100A9 null mice (n = 12) 3 weeks after s.c. injection of 1 x 10⁶ cells. Six of 12 S100A9 null mice showed significantly reduced tumor growth shown here. In addition, 2 of the remaining 6 S100A9 null mice completely rejected the tumors. B, CXCL1 in sera of wild-type and S100A9 null mice before and 3 weeks after MC38 tumor growth. C, quantitation of CD11b+ cells costaining with Gr1 or GB3.1 glycans or RAGE from bone marrow of MC38 tumor-bearing wild-type mice. D, tumors were examined for infiltrating macrophages and tumor endothelial cells by immunohistochemical staining for S100A9+ cells, CD11b+Gr1+ cells (merged images of Alexa-488-stained CD11b+ cells and Alexa-594-stained Gr1+ cells showing double-positive yellow cells), and CD31+ and F4/80+ cells (200×). E, representative sections showing CD11b+Gr1+ cells in premetastatic livers of tumor-free and tumor-bearing mice. F, quantitation of average number of CD11b+Gr1+ cells in premetastatic livers in 3 high power fields.
and lungs induced the accumulation of CD11b⁺Gr1⁺ cells, we injected separate groups of wild-type mice with MC38 cells stably expressing GFP. No GFP⁺ cells were detected in lungs or livers at 21 days after tumor initiation (not shown). MC38 tumor challenge in mice lacking S100A8/A9, or wild-type mice treated with mAbGB3.1 significantly diminished accumulation of CD11b⁺Gr1⁺-positive niches in liver (Fig. 6E and F). This finding is consistent with the studies of Hiratsuka and colleagues, who showed S100A8/A9 promote the formation of premetastatic niches in distal organs in response to primary tumors (24).

The liver is the primary site for colorectal carcinoma metastasis. Because the CAC model and ectopic MC38 tumor models did not show any evidence of distal metastasis, we chose a liver metastasis model to further understand the role of S100A8/A9- and S100A8/A9-induced proteins in promoting metastasis. We injected S100A9 null mice and age-matched C57BL/6 wild-type mice with 1 × 10⁶ MC38 cells by the intrasplenic route. MC38 cells generated tumors within the spleen (primary) and in the liver (metastasis). Multiple hepatic tumor nodules, detectable by gross inspection, were evident by 2 weeks. Livers were isolated and the incidence of hepatic metastases was evaluated. Livers from S100A9 null mice showed significantly reduced numbers of metastatic tumors, smaller tumor foci, and decreased tumor-occupied area compared with livers from tumor-bearing wild-type mice (Fig. 7). These results further indicate that S100A8/A9 play a critical role in promoting metastasis.

Taken together, our observations strongly support the notion that S100A8/A9 activate signaling pathways that promote tumor growth and metastasis by inducing expression of multiple downstream protumorigenic effector proteins and suggest that strategies that target S100A8/A9 in the tumor microenvironment could provide effective therapeutic approaches to treating patients with colorectal cancer.

**Figure 7.** S100A9 null mice exhibit reduced metastatic tumors. 
A, representative livers from wild-type and S100A9 null mice 2 weeks after intrasplenic injection of MC38 cells. Arrows indicate visible tumors. B, histology of representative livers stained by H&E (25× magnification). C, numbers of metastatic nodules in the livers, and total tumor area represented as % of liver tissue, 2 weeks after intrasplenic injection of MC38 cells (wild-type, n = 6) and S100A9 null mice (n = 5).
Discussion

Cells of the tumor microenvironment contribute to tumor growth and metastasis through complex interactions with tumor cells (58–60). The presence of S100A8/A9 in many human tumors, along with recent recognition of their roles in tumorigenesis and MDSC accumulation, warrants a more detailed understanding of the molecular mechanisms involved in their interactions within the tumor microenvironment. Our earlier studies provided evidence that S100A8/A9 promote accumulation of MDSC (7). Here we show that S100A8/A9 expressed by myeloid cells interacts with RAGE and carboxylated glycans expressed on colon tumor cells promoting intracellular signaling pathways and protumorigenic gene expression and that S100A9 null mice show reduced tumor growth and metastasis, thus defining yet another novel role for S100A8/A9 in tumor progression.

Although many studies implicate both TLR4 and RAGE in S100A8/A9-mediated pathologic effects, the relative contribution of each receptor to downstream effects is unknown. Based on our earlier studies and immunoprecipitation results shown here, we surmise that RAGE is the principal receptor of S100A8/A9 on tumor cells, and this is consistent with the finding that S100A8/A9-mediated responses in human tumor cells involves RAGE (21, 23). However, studies implicating S100A8/A9 in endotoxin-induced lethality and systemic autoimmunity show that TLR4, rather than RAGE, could play a more prominent role as receptor for these ligands on macrophages (17, 18). This suggests that cell types and pathologic settings could dictate which receptor predominates. Besides cell types, the differential effects could also be mediated by carboxylated glycans, which are expressed on RAGE and not on TLR4. Also, TLR4 is only functional active in the presence of myeloid differentiation factor-2 (MD2) protein for both LPS and S100A9-mediated interactions on macrophages (17). TLR4 expressed on MC38 cells is functional, as it has been shown to respond to LPS, as determined by LPS-induced expression of IL-6 by MC38 cells, which is reduced upon TLR4 gene silencing (61).

The activation of RAGE-mediated signaling pathways could also depend on the tumor cell involved. We found that S100A8/A9 induces RAGE and carboxylated glycandependent phosphorylation of ERK1/ERK2 and SAPK/JNK MAPK in colon tumor cells, but we did not observe significant phosphorylation for p38. In contrast, in human prostate and breast cancer cells, S100A8/A9 activate p38 but not SAPK/JNK (21, 23). In this context, it is interesting that S100A8/A9 activate SAPK/JNK in macrophages through TLR-dependent pathway (62). In support of our finding, it was recently shown that treatment of tumor cells with a JNK inhibitor blocked RAGE ligand-induced cellular invasion (63). p38 and SAPK/JNK MAPK proteins are known to function in cell context and cell type–specific manner to coordinate signaling pathways mediating tumor cell proliferation, survival, and migration and may even exert antagonistic effects, depending on signal duration and cross-talk with other signaling pathways (64). Their expression is altered in many human tumors, and it is therefore important to consider the tumor type before modulations of the pathways are attempted for therapeutics.

S100A8/A9 binding to colon tumor cells stimulates RAGE and carboxylated glycan–dependent activation of NF-κB pathway. NF-κB provides a critical link between inflammation and cancer (43, 44). As the binding of S100A8/A9 to cells stimulates NF-κB transcription, and proximal promoter regions of S100A8 and S100A9 have binding sites for NF-κB (10), ligation of cell surface receptors by S100A8/A9 in inflammation could lead to a positive feedback loop and sustained cellular activation promoting tumor development. In support of this, S100A8/A9 proteins have been identified as novel NF-κB target genes in hepatic carcinoma cells during inflammation-mediated liver carcinogenesis (65).

Our gene expression analysis revealed for the first time the molecular signature of S100A8/A9 activation in tumor cells. Some of the genes that are activated represent known NF-κB target genes and are directly associated with tumorigenesis. Most notable are the chemokines CXCL1 (GROα or KC), CCL2 (MCP-1), CCL5 (RANTES), and CCL7 (MCP-3). Although many previous studies have focused on the role of chemokines in immune responses, recent studies show that they also promote chemotaxis and leukocyte recruitment to tumors, angiogenesis, bidirectional cross-talk between tumor cells and tumor-associated fibroblasts, tumor invasion, and migration of tumor cells to distal organs (47, 48, 66). CXCL1, CCL2, and CCL5 have been also identified in human colorectal tumors and expression correlates with poor prognosis (46, 67, 68). CCL2 is a crucial mediator of CAC in mice (69) and CXCL1 mediates proangiogenic effects of PGE2 in colorectal cancer (70).

More recent studies show that the expansion of CD11b⁺Gr1⁺ MDSC in premetastatic sites in liver in response to intraabdominal tumors is contingent upon the expression of CXCL1, and self-seeding of circulating tumor cells promote tumor growth, angiogenesis, and tumor recruitment through mediators such as CXCL1 (57, 71). We found that CXCL1 is secreted by colon tumor cells in response to interaction with S100A8/A9 and is upregulated in colon tumors. It is elevated in sera of not only the MC38 tumor-bearing mice but also in mice with colitis-induced tumors within 12 weeks of AOM-DSS treatment, at which time point the tumors are early, well-contained, and noninvasive, suggesting that CXCL1 could provide an early marker of metastatic tumor progression. We also found that tumor-bearing mice lacking S100A8/A9 show marginal or no elevation of CXCL1 and significantly diminished CD11b⁺Gr1⁺ cells in tumors and premetastatic niches in liver and lungs.

In addition, we found other new S100A8/A9-induced genes that are implicated in tumor progression. Zc3h12a, which encodes a zinc finger protein, is an RNase and a downstream effector of CCL2-mediated angiogenesis (72). Enpp2 encodes autotaxin, a lysophospholipase D enzyme that hydrolyzes extracellular lysophospholipids to produce lysophosphatidic acid (LPA). LPA receptors are
overexpressed in many tumors (50), and LPA2 receptor knockout mouse shows markedly reduced tumor incidence and progression of colon adenocarcinomas associated with reduced tumor-infiltrating macrophages (73). More recently, Liu and colleagues showed a causal link between autotaxin-LPA receptor signaling and mammary tumor progression (74). Autotaxin is also implicated in the formation of invadopodia by various human cancer cell types (75). Slpi encodes a secretory leukocyte peptidase inhibitor that is upregulated in tumors. It protects epithelial tissues from serine proteases and has been implicated in wound healing (76). Neutrophil gelatinase–associated lipocalin-2 encoded by NF-kB inducible Lcn2 gene has paradoxically both pro- and antitumor effects (52). Pif2 encodes prolif-erin-2, a hematopoietic stem cell growth factor associated with angiogenesis and wound healing (77, 78).

The induction of these downstream effector genes in tumors would thus greatly amplify tumor growth, migration and invasion, induction of myeloid cells, and metastatic progression promoted by S100A8/A9. Consistent with this, we found that S100A9 null mice showed markedly reduced tumor incidence and progression of AOM-DSS induced colon adenomas and reduced ectopic MC38 tumor growth and tumor metastasis. Because CD11b+Gr1+ cells in the tumor-bearing mice are MDSC, it is likely that reduced tumor growth and metastasis in S100A9 null mice are due to combined effects of lack of immune suppression and reduced induction of protumorigenic genes. The effects of S100A8/A9 in tumorigenesis in the AOM-DSS model could be mediated through RAGE and carbohydrate glycans, as we earlier showed that RAGE null mice and wild-type mice receiving mAbGB3.1 treatment showed reduced AOM-DSS induced tumor incidence. However, the contribution of TLR4 in S100A8/A9-mediated effects in tumors cannot be overlooked, because TLR4 null mice are protected markedly from CAC (79) and TLR4 mediates the formation of premetastatic niches promoted by S100A8/A9 (32).

Colorectal cancer is one of the most common malignancies affecting both sexes and a common cause of mortality worldwide. Each year about 50,000 people die from the disease in the United States alone. Our present findings, along with earlier studies, show that S100A8/A9 function at multiple stages in disease progression. S100A8/A9, their receptors and signaling pathways therefore provide important targets for development of pharmacological interventions and for the identification of early-stage disease biomarkers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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41. Earl TM, Nicoud IB, Pierce JM, Wu K, Majoras NE, Rubin JE, et al. Silencing of TLR4 decreases liver tumor burden in a murine model of
Correction: S100A8/A9 Activate Key Genes and Pathways in Colon Tumor Progression

In this article (Mol Cancer Res 2011;9:133–48), which was published in the February, 2011 issue of Molecular Cancer Research (1), an incorrect microarray data accession number was published. The correct accession number is GSE26359.

Reference

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